NOTES ON A TOXIN FROM *GYMNODINIUM BREVE*

By

THEODORE J. STARR

[Reprinted from TEXAS REPORTS ON BIOLOGY AND MEDICINE, Volume 16,
Number 4, pages 500–507, Winter, 1958]

NOTES ON A TOXIN FROM *GYMNODINIUM BREVE*

THEODORE J. STARR[a]

Catastrophic fish mortalities associated with algal blooms have occurred at various times throughout the world (Connell and Cross, 1950; Ballantine and Abbott, 1957). In the Gulf of Mexico, blooms of *Gymnodinium breve* (Davis, 1948) accompanied massive fish kills which were reported along the west coast of Florida (Gunter *et al.*, 1948), the southeast coast of Texas, and the northeast coast of Mexico (Wilson and Ray, 1956). *G. breve* appears to be widely but sparsely distributed in the Gulf of Mexico. A critical combination of chemical and physical factors sets off blooms (Ketchum and Keen, 1948; Gunter *et al.*, 1948; Hela, 1955; Chew, 1955).

Until recently, studies of the metabolism, physiology, and toxin of *G. breve* were hampered because of the fragile nature and fastidious growth requirements of this organism. Wilson and Collier (1955) developed an enriched sea-water medium which supported growth and serial transfer of unialgal cultures of *G. breve*. Their mass cultures killed fish. Ray and Wilson (1957) then isolated *G. breve* and showed that pure cultures killed fish. They reconfirmed the toxicity of unialgal cultures.

This study describes some procedures for the bioassay of the toxin in unialgal cultures of *G. breve* and some properties of crude toxin preparations.

Materials and Methods

Growth of mass cultures. Unialgal mass cultures of *G. breve* were grown in 20 liter carboys containing 15 liters of the sea-water medium described by Wilson and Collier (1955) with (a) half the amount of soil extract, (b) 3 x the final concentration of $Na_2S \cdot 9H_2O$, and (c) omission of EDTA. Good growth (1 to 3 million living cells per liter) was obtained in 4 to 8 weeks; toxicity experiments were then conducted.

Bioassay organisms. In early experiments the guppy, *Lebistes reticulatus*, was used to compare the potencies of different preparations of *G. breve*. The guppies, bred in quantity in laboratory tanks, were available throughout the year. Although the guppies did not breed in waters of high salinity (approximately 2.8 to 3.3 per cent) they survived at these salinities and could be transferred from fresh water without adverse effects. This ability to withstand high salinities was a prerequisite for its use in bioassays since most of the tests were made in the sea-water medium (salinity, 2.8 to 3.3 per cent, pH 7.9 to 8.2) described above.

In later experiments mullet, *Mugel cephalus*, were used. Small mullet (approximately 2.8 to 3.0 cm.) were readily available in the immediate vicinity of December through June. As they could not be maintained under our laboratory conditions for more than a week without loss of vitality, they were caught with a dip net when needed (several hundred at a time from one school) and used within 1 to 3 days. Small mullet proved sensitive to changes in salinity. Those caught in low-salinity waters (0.3 to 0.8 per cent) were gradually adjusted to higher salinities (2.8 to 3.3 per cent) by the dropwise addition of saturated NaCl to their holding tanks (100 liters) over a period of 24 to 36 hours. Usually this adjustment was unnecessary.

Responses of bioassay organisms to the toxin. Most of our toxin preparations from unialgal mass cultures (1.5 million cells per liter) killed mullet within 2 to 4 minutes. As the preparations were diluted, the symptoms of distress and time to death were prolonged. Mullet showed the following: within 30 seconds a violent twisting and turning accompanied by cork-screw type movements; then contractions and tail curvature at intervals of 10 to 20 seconds; within 1 to 2 minutes equilibrium is lost and fish may turn upside-down or on its side; opercular movements are irregular and slow; little response to probing and they may remain quiescent; immediately before death a violent burst of activity occurs and the fish dies with mouth and opercula opened. Once equilibrium is lost, removal to non-toxic water does not aid recovery.

Toxin preparations which kill mullet in 2 to 4 minutes kill guppies in 8 to 15 minutes. The response of the guppy is sluggish compared to the mullet but the symptoms are similar. It remains to be seen if the toxin acts alike in both fish.

These symptoms are remarkably similar to those described by Abbott and Ballantine (1957) for *Gobius flavescens* (goby) in a toxic preparation of *Gymnodinium veneficum* (Ballantine, 1956).

Bioassay techniques using mullet. One liter aliquots of a mass culture (final pH 8.15, salinity 3.3 per cent, 7 weeks old) having a living cell count of 1.5 million per liter were frozen overnight in flasks. The contents were thawed in a water bath at room temperature (25±2° C.) and filtered through an AA millipore filter. Dilutions were made from the cell-free preparation with sea-water medium (pH 7.9, salinity 3.3 per cent) to give the following final concentrations: (ml. *G. breve* preparation per 100 ml. final solution) 0.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 75.0 and 100.0. One hundred ml. of each dilution were put into 250 ml. beakers. Mullet were washed in the uninoculated sea-water medium for 15 to 30 minutes. At zero time, fish were added to each dilution and death times recorded.

Results

Preliminary experiments with guppies and mullet indicated that living cultures of *G. breve* were less toxic than killed ones.

In many instances, our test organisms remained active for approximately one hour in a culture of live *G. breve*. However, aliquots of the culture, previously treated to destroy *G. breve*, killed both assay organisms in a matter of minutes. Gentle heating, quick freezing, pH changes, vacuum filtration, changes in osmotic pressure, and addition of certain heavy metals to aliquots of the culture, all increased toxicity.

Standard bioassay death curve for mullet. The standard bioassay death curve (Fig. 1) was made with a cell-free preparation. It represents a composite of 4 experiments with a total of 12 to 16 fish for each dilution tested.

Minimum and maximum death times are indicated by the extremes of the vertical lines. The points at which the assay curve passes through the vertical lines are the average death times of mullet in the indicated concentration of the *G. breve* preparation. As shown, undiluted cell-free preparations (100 per cent) killed mullet within 2 to 4 minutes; with increasing

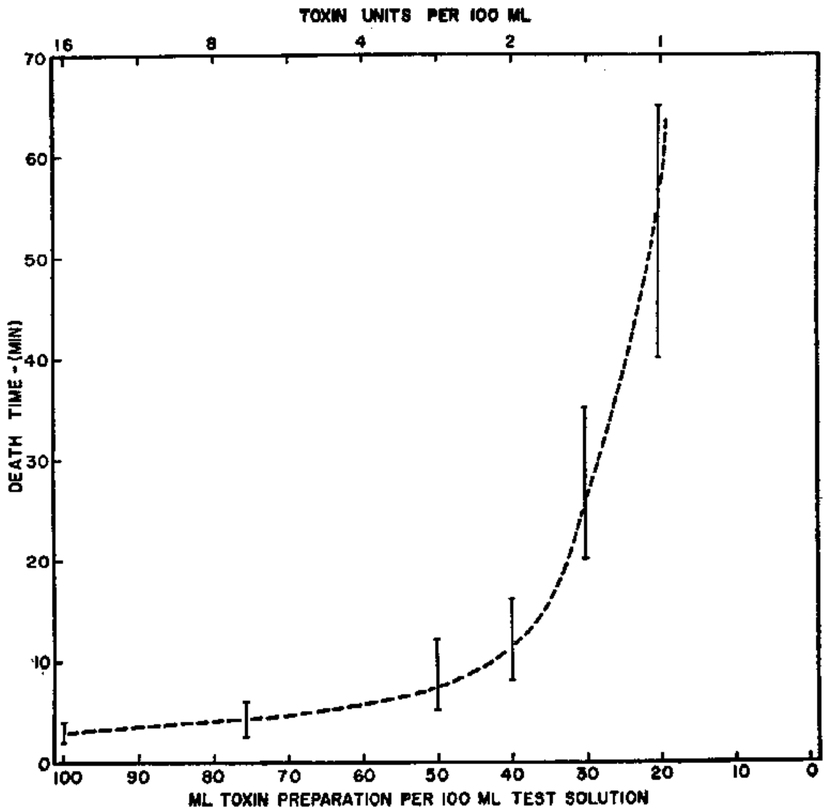

FIG. 1. Response of mullet to *Gymnodinium breve* toxin dilutions. Arbitrary toxin units (TU) defined in text.

dilutions, average death times were increased. In a 20 per cent dilution, mullet were killed in approximately 60 minutes. A 10 per cent dilution proved toxic in 4 to 8 hours and sometimes not at all. At concentrations below 10 per cent and in controls, mullet were still alive after 72 hours at which time the experiment was discontinued. Death times at specific concentrations increased with decreasing dilutions of the original preparation as indicated by the heights of the vertical lines in figure 1. Thus, at concentrations representing 100, 75, 50, 40, 30, and 20 per cent of the cell-free preparation, the death range for different mullet was 2, 4, 7, 8, 15, and 25 minutes, respectively. At concentrations representing 10 per cent of the original preparation which at times would kill mullet within 4 to 8 hours or not at all, the variability in death time was too great for inclusion in the standard curve.

Comparison of Abbott and Ballantine's (1957) data with ours suggests a similarity of action between the *G. veneficum* and *G. breve* toxins. They reported that a strong toxic culture of *G. veneficum* killed gobies in 5 to 6 minutes. Our usual cell-free preparations killed mullet within 2 to 4 minutes. Preparations diluted to 60 per cent killed within 5 to 6 minutes. Occasionally concentrated preparations were obtained which killed within 30 to 60 seconds.

The toxin unit. One *G. breve* toxin unit (TU) is defined arbitrarily as the amount of toxin per 100 ml. of sample which under standard conditions will kill *Mugel cephalus* in 60 ± 15 minutes and a two-fold dilution of which may kill within 4 to 8 hours or not at all. The standard conditions include: mullet 2.5 to 3.0 cm. in total length, 100 ml. of test solution contained in 250 ml. beakers, controlled temperature $25 \pm 2°$ C., immediate use of test solutions (some activity is lost on standing for prolonged periods), and appropriate salinity and pH controls.

Effect of temperature on toxicity. In preliminary experiments, aliquots of living cultures of *G. breve* exposed to lethal temperatures below 10° C. or above 37° C. proved more toxic than the original culture. Freezing-thawing or a 1 to 3 minute exposure at 50° C. were most effective in this respect.

Unialgal mass cultures of *G. breve* and uninoculated control medium were divided into liter aliquots and frozen overnight. After thawing in a water bath at 25° C., aliquots were assayed

and contained 16 TU (killed mullet in 2 to 4 minutes). Other aliquots were filtered through AA millipore filters. Residues (cell debris) were reconstituted with control medium to concentrations representing 20 x the original culture. Little or no activity was detected. The supernate or cell-free preparation contained most of the original activity (16 TU).

The effect of temperature on cell-free preparations containing 16 TU was tested in two series of experiments. In the first, a 2 liter aliquot was placed into a 4 liter flask and heated on a hot plate. Aliquots of 100 ml. were removed at temperatures of 25, 50, 65, 80, and 100° C., cooled immediately to 25° C. in an ice bath, and assayed. Little or no activity was lost from a cell-free preparation heated to 80° C. (Table 1). Approximately 1 TU

TABLE 1

Effect of increasing temperatures on the toxicity of cell-free preparations of Gymnodinium breve *with mullet the assay organism*

Temp. ° C.	Death Time Range (min.)
25	3–4
50	3–4
65	4–7
80	3–8
100	47–80

remained in aliquots of cultures heated to 100° C. In the second series, 2 liter aliquots of a cell-free preparation (16 TU) were placed in constant temperature baths at 25, 45, 55, and 100° C. After reaching the indicated temperatures, 100 ml. aliquots were removed at various times (Table 2), cooled to 25° C. in an ice bath, and assayed. Only 1 to 2 TU could be detected in those samples heated to 45° C. for about 4 hours or to 55° C. for 2 hours. No toxicity could be detected in samples held at 100° C. for 5 minutes.

Effect of pH on toxicity. The influence of pH on the toxicity of a living *G. breve* culture and on the toxicity of a cell-free preparation was tested. Aliquots of a culture (5 weeks old, final pH 8.1, salinity ca. 3.0 per cent, living cell count 1.2 million per liter) and uninoculated control medium were adjusted with 5 N HCl or 5 N NaOH to pH 3.3, 5.4, 7.0, 8.1, and 9.5 (precipitates occurred at the latter pH). At pH 3.3, 5.4, and 9.5, *G. breve* cells were destroyed and mullet were distressed in 17 to 19 minutes. At the original pH 8.1, *G. breve* remained active for an extended period

TABLE 2

Effect of exposure time at elevated temperatures on the toxicity of cell-free preparations of Gymnodinium breve *with mullet the assay organism*

Time (min.)	Average Death Time (min.) 25° C.	45° C.	55° C.	100° C.
0	3.5	3.5	3.5	64
5	..	..	3.5	still alive 72 hr.
15	..	..	4.0	still alive 72 hr.
25	..	3.5	..	still alive 72 hr.
30	3.5	..	15	.....
40	..	..	22	still alive 72 hr.
60	3.5	..	..	.....
65	..	3.5	..	.....
90	..	..	36	.....
120	4.5	..	..	.....
137	..	..	190	.....
150	..	42	..	.....
180	..	..	250	.....
240	4.0	..	...	.....
285	..	120	...	.....

and mullet were distressed in 45 minutes. All fish in pH control vessels appeared normal after 2 hours except for those held at pH 3.3 which showed signs of distress.

A similar test was made with a cell-free preparation containing 4 TU (killed mullet in 5 to 10 minutes). Aliquots were adjusted to pH 5, 6, 7, 8, and 9. The average death times for mullet in solutions at these pH levels were 5, 4, 8, 9, and 7 minutes, respectively. Other aliquots were adjusted to the same pH levels, readjusted to pH 8.0, and then tested. There was no apparent change in toxicity of the original preparation due to pH changes. Controls were still alive after 24 hours.

Within the range of values tested, pH does not modify the activity of the toxin as it affects mullet. However, a radical pH change which destroys *G. breve* enhances the toxicity of the original culture.

The effect of heavy metals on toxicity. The effect of heavy metals on the toxicity of a living culture of *G. breve* was tested. Salts of Sr, Zr, Hg, Ag, and Cu (final concentrations 5 ppm.) were added to 100 ml. aliquots of a culture. Distress times of the guppy and the state of activity of *G. breve* were recorded (Table 3). Hg, Ag, and Cu were lethal to *G. breve* within 7 minutes and guppies showed signs of distress within 13 to 18 minutes. Zr and Sr were less effective in this respect; *G. breve* remained active for ca. 60 minutes and guppies showed signs of distress within 111

TABLE 3

Effect of heavy metals on Gymnodinium breve *and on the toxicity of a living culture with guppy the assay organism*

Metal (5 ppm.)	State of *G. breve*	Average Distress Time for Guppy
Control, no addition	dying—100 min.	120 min.
Sr, as $SrCl_2 \cdot 6H_2O$	dying— 60 min.	183 min.
Zr, as $ZrOCl_2 \cdot 8H_2O$	dying— 60 min.	111 min.
Hg, as $HgCl_2$	dead — 7 min.	18 min.
Ag, as $AgNO_3$	dead — 7 min.	13 min.
Cu, as $CuSo_4 \cdot 5H_2O$	dead — 7 min.	13 min.

to 183 minutes. In controls containing no added metals, guppies were distressed in 120 minutes at which time the numbers of living *G. breve* had decreased considerably. These results indicated that metals which destroy *G. breve* effectively enhance the toxicity of the culture.

Attempts to concentrate the toxin. These experiments indicated that the toxin is confined mainly within the living organism. Attempts were then made to harvest intact cells for possible concentration and isolation of the toxin. Vacuum filtration and slow centrifugation were ineffective because the fragile *G. breve* was destroyed with release of the toxin. Intact cells were absorbed on filter paper but the activities of the reconstituted residues decreased with increasing volumes of the original culture. Thus, the filtrate of several liters of culture contained most of the original activity. However, the residue from smaller volumes (50 to 200 ml.) was absorbed on the filter and the toxin could be eluted from it with little loss of the original activity.

Charcoal and alpha cellulose at various acid and alkaline pH's destroyed or removed the toxin from living cultures and cell-free preparations. Attempts to elute the toxin with organic solvents and buffers failed. Aliquots of a living *G. breve* culture in cellulose dialyzing tubing were dialyzed against several changes of distilled water. Samples removed from the bag after 6 hours (*G. breve* was destroyed) contained most of the original activity.

Summary

Some properties of a toxin in unialgal mass cultures of *Gymnodinium breve* are described. Mullet and guppies were used to compare the potency of different toxin preparations. *G. breve* is extremely sensitive to chemical and physical manipulation and the toxicity of a living culture is increased by agents which destroy *G. breve*. Such observations indicate that the toxin is largely within the cell. However, the fragility of *G. breve* has rendered fruitless attempts to concentrate intact cells as a means of concentrating the toxin.

In the absence of further information, it is difficult to compare the toxin of *Gymnodinium veneficum* (Abbott and Ballantine, 1957) with the toxin of *G. breve*. They are probably similar. Although the *G. veneficum* toxin has been described as an exotoxin (secreted into the water), it is difficult to tell whether *G. breve* produces a true exotoxin (freely secreted or excreted into the environment by living cells) or an endotoxin (liberated from the cells by lysis or autolysis).

REFERENCES AND NOTES

Abbott, B. C., and D. Ballantine: The toxin from *Gymnodinium veneficum* Ballantine. *J. Marine Biol. Assoc. United Kingdom*, 36: 169–189, 1957.

Ballantine, D.: Two new marine species of *Gymnodinium* isolated from the Plymouth area. *J. Marine Biol. Assoc. United Kingdom*, 35: 467–474, 1956.

Ballantine, D., and B. C. Abbott: Toxic marine flagellates; their occurrence and physiological effects on animals. *J. Gen. Microbiol.*, 16: 174–281, 1957.

Chew, F.: Red tide and the fluctuation of conservative concentrations at an estuary mouth. *Bull. Marine Sci. Gulf and Caribbean*, 5: 321–330, 1955.

Connell, C. H., and J. B. Cross: Mass mortality of fish associated with the protozoan *Gonyaulax* in the Gulf of Mexico. *Science*, 112: 359–363, 1950.

Davis, C. C.: *Gymnodinium brevis* sp. nov., a cause of discolored water and animal mortality in the Gulf of Mexico. *Bot. Gaz.*, 109: 358–360, 1948.

Gunter, G., R. H. Williams, C. C. Davis, and F. G. W. Smith: Castrophic mass mortality of marine animals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. *Ecol. Monogr.*, 18: 309–324, 1948.

Hela, I.: Ecological observations on a locally limited red tide bloom. *Bull. Marine Sci. Gulf and Caribbean*, 5: 269–291, 1955.

Ketchum, B. H., and J. Keen: Unusual phosphorus concentrations in the Florida Red Tide sea waters. *J. Marine Res.*, 7: 17–21, 1948.

Ray, S. M., and W. B. Wilson: Effects of unialgal and bacteria-free cultures on *Gymnodinium brevis* on fish. *Fishery Bull. No. 123*, 57: 469–495, 1957.

Wilson, W. B., and A. Collier: Preliminary notes on the culturing of *Gymnodinium brevis* Davis. *Science*, 121: 394–395, 1955.

Wilson, W. B., and S. M. Ray: The occurrence of *Gymnodinium brevis* in the western Gulf of Mexico. *Ecology*, 37: 388, 1956.

[a] From U.S. Fish and Wildlife Service Gulf Fishery Investigations, Galveston, Texas. Present address: The University of Texas Medical Branch, Department of Preventive Medicine and Public Health, Virus Research Laboratory, Galveston, Texas. Accepted for publication, June 24, 1958.